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Immuno-chromatography based rapid detection of canine distemper in dogs and its comparison with RT-PCR

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Abstract

The present study was planned to confirm the clinical diagnosis of canine distemper in dogs with reverse transcriptase polymerase chain reaction (RT-PCR) and its comparison with lateral flow assay based immuno-chromatographic (IC) technique. Fifty clients owned dogs having clinical signs suggestive of respiratory, skin or nervous form of canine distemper were included in the study. An immuno-chromatography-based test was applied using serum to screen each of the suspected dog. In addition to serum, nasal discharges of 15 cases and ocular discharges of 10 samples were used to diagnose canine distemper. Screening with IC kit revealed 72.00% serum samples positive, 66.66% ocular and 50.00% nasal samples were found positive for antigen. The RT-PCR targeting N gene of canine distemper virus was used for the molecular diagnosis of canine distemper. Out of 50 blood samples tested with RT-PCR assay, 38 (76.00%) samples were positive showing characteristics band of 287bp. Statistical comparison of IC Kit (serum samples) results with RT-PCR results comparison showed that IC kit was 52.00% accurate with 36.84% sensitivity, 100% specificity, positive predictive value of 93.33% and negative predictive value of 31.43%. In the current study it was observed that the IC test was rapid, quick and specific but was found to be less sensitive compared to RT-PCR.

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Introduction

Canine distemper (CD) is highly contagious viral disease initially described as an infectious disease of domestic dogs only, however, gradually is recognized as a global multi host disease infecting wide range of carnivore species and causing mass mortalities.[1] Canine distemper virus (CDV) is a pantropic virus associated with multiple cell tropisms leading to systemic infection including respiratory, urinary, lymphatic, cutaneous, skeletal and central nervous system diseases.[2] It is a large, enveloped, non-segmented negative sense, single stranded RNA virus of genus Morbillivirus in *Paramyxoviridae* family. Clinical signs of distemper in dogs are often overlapping and vary depending upon the involvement of multiple bodily systems, therefore, it is difficult to confirm the disease.

An early detection of a deadly disease of contagious nature as of CD, can ascertain steps to check further spread of disease by following appropriate measures including isolation and quarantine of affected dogs and simultaneously initiating the suitable treatment for the same. Rapid diagnosis of infection had been attempted in past through detection of antibodies and antigen.[3] However, serological tests suffer a limitation of not diagnosing the infection in asymptomatic and low viremic cases. Definitive diagnosis of infection can be done by virus isolation and molecular diagnostic tests. However, isolation of virus is tedious and time consuming.[4] Various molecular tests including conventional polymerase chain reaction (PCR), nested and real-time reverse transcription polymerase chain reaction assays that offer high degrees of detection sensitivity and specificity have also been developed for CDV.[5] Despite the known advantages of various molecular tests, immuno-chromatography based lateral flow assays have a distinct edge of early detection of the disease, thereby, paving the way for initiation of specific therapy and future advisement to the pet parents. Keeping in view the above facts, the present study was planned to compare sensitivity, specificity and accuracy of immuno-chromatography-based antigen detection using serum, nasal and ocular discharges to those of reverse transcription-PCR assay.

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Materials and Methods

Sample collection: The present study was conducted on 50client owned dogs showing respiratory and nervous signs suggestive of CD which were brought to the Veterinary Clinical Complex, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana, India, for the diagnosis and therapeutic management. Serum and blood samples were collected from all the dogs considered under the present study. In addition to serum samples, 15 nasal discharges and 10 ocular discharges were tested by immuno-chromatographic (IC) kit (FASTest DISTEMPER strip; Diagnostik Megacor, Hoerbranz, Austria).

Rapid detection of CDV antigen using Immuno-chromatography based lateral flow assay: For initial screening and qualitative detection of CDV antigen, commercially available immuno-chromatography test kits (FASTest DISTEMPER strip, Diagnostik Megacor) were used. The test was conducted with serum samples, nasal and ocular secretions following the manufacturer's instructions provided along with the kit. Positive test result was marked when weak to strong and well defined pink purple colored test line, and control line was observed while appearance of only pink-purple control line was marked as negative test.

Detection of CDV using RT-PCR assay. Viral RNA extraction using TRIzol method. Blood samples collected from suspected dogs were subjected to RNA extraction. Briefly, 400 µL of the blood sample was poured in 2.00 mL Eppendorf tube and treated with 800 µL of TRIzol reagent followed by vigorous vortexing and then kept at room temperature for 15 min. To this, 200 µL of chloroform was added followed by vigorous vortexing to avoid insoluble aggregates and then kept at room temperature for 15 min. The mixture was centrifuged at 12,000 rpm for 15 min. at 4.00 ˚C and the upper aqueous phase was separated in 1.50 mL Eppendorf tubes without disturbing the organic phase and inter phase. The micro centrifuge tube was inverted four to five times after adding equal volume of isopropanol and kept overnight at – 20.00 ˚C. The RNA was pelleted by centrifugation at 12,000 rpm for 15 min at 4.00 ˚C. The supernatant was discarded and after one washing with 1.00 mL of 70.00% chilled ethanol, RNA was pelleted by centrifugation at 12,000 rpm for 15 min and air dried for 2 to 3 hr. Pellet was directly dissolved in nuclease free water and was used further for first strand cDNA synthesis. Similarly, nasal and ocular discharges in PBS were used for RNA extraction. RNA extracted from commercially available live attenuated multi component vaccine for CDV was considered as positive control for standardization of assay.

cDNA synthesis. The cDNA of extracted RNA samples was prepared using Revert aid first strand cDNA synthesis kit by Thermo Fischer Scientific (Waltham, USA). A 20.00 µL reaction consisting of 8.00 µL master mix (4.00 µL Reverse Transcriptase buffer (5.00 X), 2.00 µL dNTPs, 1.00 µL RT enzyme; 1.00 µL RiboLock), 5.00 µL of RNA template, 1.00 µL of Random Hexamer and 6.00 µL nuclease-free water to make reaction volume to 20.00 µL. Initially mixture was preheated for primers annealing at 25.00 ˚C for 10 min, followed by reverse transcription step carried out at 42.00 ˚C for 60 min in a programmable 96 well thermal cycler. The reverse transcriptase was heat inactivated at 70.00 ˚C for 5 min. The cDNA was stored at – 20.00 ˚C till further use.

Primer pair and cyclic condition for standardization. pCD/N/(F): ACA GGA TTG CTG AGG ACC TAT and pCD/N/(R) CAA GAT AAC CAT GTA CGG TGC primer pair previously used and published by Frisk *et al.*[6] was procured from Sigma Aldrich (St. Louis, USA). Reverse transcriptase polymerase chain reaction was performed in thermal cycler (Veriti; Thermo Fischer Scientific) in 12.50 µL reaction containing 3.00 µL of template DNA, 6.25µLof Master mix 2.00 X concentration (Thermo Fischer Scientific) containing 15.00 Mm MgCl2, 1.00 µL of dNTPs mix (10.00 mM), 1.00 µL of Taq DNA polymerase (1.00 U per µL), 0.50 µL each of forward and reverse primers (10.00 pM concentration) and 2.25 µL of nuclease free water. Cyclic conditions for pCD/N primers included one cycle of initial denaturation at 95.00 ˚C for 1 min, followed by 40 cycles of denaturation at 95.00 ˚C for 30 sec, annealing at 59.50 ˚C for 30 sec and extension at 72.00 ˚C for 1 min and final extension for 15 min.

Agarose gel electrophoresis. The PCR products were analyzed in 1.50% agarose gel containing 0.50 µg mL^{-1} ethidium bromide. For size estimation of amplicons, standard molecular size marker (Gene Ruler 100 bp DNA Ladder; Thermo Fischer Scientific) was run parallel to the amplified products in agarose gel electrophoresis. Electrophoresis was carried out at 5.00 V cm^{-1} of gel in 1.00 X Tris-acetate-EDTA running buffer for 80 min in submarine electrophoresis apparatus. The gel was visualized under ultra-violet transilluminator provided in gel documentation system (Bio-Rad, Hercules, USA) and documented by photography for further analysis. Determination of sensitivity, specificity and accuracy of immuno-chromatography were based on pen side kit and RT-PCR. Results of immuno-chromatography-based pen side kit were compared to those of RT-PCR to determine the sensitivity, specificity and accuracy of IC based kit.

$$Sensitivity = \frac{True\ Positive}{True\ Positive + False\ Negative}$$

$$Specificity = \frac{True\ Negative}{True\ Negative + False\ Positive}$$

$$Accuracy = \frac{True\ Negative + True\ Positive}{True\ Negative + True\ Positive + False\ Negative + False\ Positive}$$

Results

In the present study, fifty dogs showing clinical signs suggestive of CD were screened with CD antigen based immuno-chromatography test kit where 72.00% serum samples, 66.66% ocular and 50.00% nasal samples were found positive for antigen (Fig. 1) with immuno-chromatography-based test. In addition, five ocular and three nasal discharges were found positive where the serum samples tested negative. The RT-PCR conducted with fifty suspected blood samples of dogs in the present study confirmed thirty-eight dogs to be positive (76.00%) for CD (Fig. 2). Statistical comparison of IC to RT-PCR to blood/serum, ocular and nasal samples, respectively, showed 36.84, 100, 52.00; 60.00, 30.00, 40.00 and 66.60, 57.10, 60.00% for sensitivity, specificity and accuracy (Table 1).

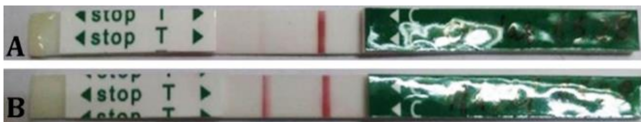

Fig. 1. Immuno-chromatography test showing positive result with **A)** serum (faint T line), and **B)** ocular discharge.

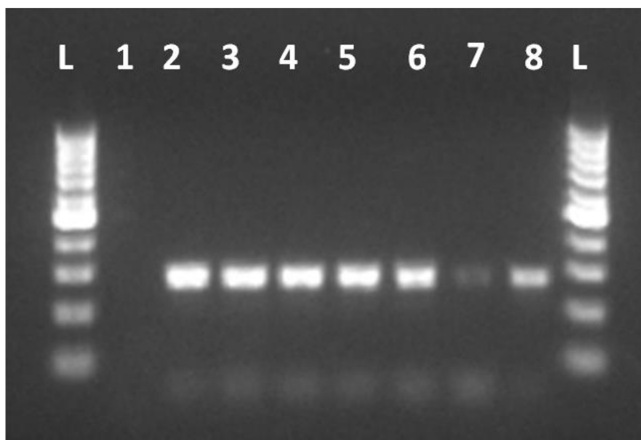

Fig. 2. Result of agarose gel electrophoresis showing polymerase chain reaction products of reverse transcription-polymerase chain reaction assay targeting canine distemper virus N gene. Lane L: 100 bp ladder. Lane 1: Negative control; Lane 2: Positive control (vaccine); Lanes 3-8 field samples (positive).

Discussion

Canine distemper infection is a highly contagious disease of dogs and other carnivores causing high morbidity and mortality rates. In the present study, lower sensitivity of IC as compared to RT-PCR could be attributed to failure of IC to detect the infected cases harboring low concentration of viremia. It is also worth mentioning here that in the present study the sensitivity and specificity of IC test compared to RT-PCR vary with the test samples used. Awad in a similar study reported 24 out of 54 dogs (44.44%) were positive for CD when their conjunctival swabs were tested using IC kits.[3] Conjunctival swabs are easy to obtain in early phase of CD infection and IC assay performed using conjunctival swabs resulted in maximum sensitivity and specificity (100 and 100%, respectively) relative to nested PCR with the similar samples.[7] Kim *et al.* was also in the view that in a suspected case of CD, animals with exuberant ocular and nasal secretions tend to provide good clinical material for screening.[4]

In contrast to the present findings, Desai *et al.*[7] found only two dogs positive by immuno-chromatography-based lateral flow test (LFA) test whereas 10 dogs were found positive in one step RT-PCR. They supported that though LFA was rapid in detection, it gave a false negative result which was attributed to the limit of detection and low device sensitivity along with low and interrupted secretion of virus which could be the reason of false negative results when nasal swabs, ocular swabs and Cerebrospinal fluid were used. Similarly, An *et al.* reported slightly lower sensitivity of LFA for detection of CDV.[8] Yang *et al.* also reported lowered detection limit of rapid IC test kits.[9] However, they observed higher detection limit of Rapid immuno-chromatographic test (RICT) kits than that of the nested RT-PCR or multiplex RT-PCR though they were in the view that the sensitivity of the RICT kits needs to be improved using antibodies that can capture all CDV genotypes.

Confirmative diagnosis is necessary to rule out different diseases as multiple clinical infections have overlapping clinical signs in dogs. Thus, it is required to perform sensitive molecular based diagnosis as it can detect even low amount of viral RNA in samples.[7] The RT-PCR has been successfully applied on various samples for diagnosis of CDV in past.[6,10-12] The use of RT-PCR with all different body fluids (serum, CSF, ocular swabs, urine and whole blood) is advocated by different authors to increase the sensitivity as the CDV RNA shows a heterogeneous distribution in different body compartments.[6,13,14] For animals with early systemic signs, including fever, prostration and inappetence, the indicated specimen of choice would be blood and/or urine where RT-PCR though

Table 1. Determination of sensitivity, specificity and accuracy of immuno-chromatography-based pen side kit and reverse transcription-polymerase chain reaction (RT-PCR) for diagnosis of canine distemper virus.

Parameters	Blood samples/serum samples (n = 50)	Nasal discharge (n =10)	Ocular discharge (n =15)
Sensitivity (%)	36.84	60.00	66.60
Specificity (%)	100	30.00	57.10
Accuracy (%)	52.00	40.00	60.00

found less sensitive than nested PCR showed excellent application for laboratory diagnostic purposes.[15] Some studies have verified that urine is a good biological sample for the detection of CDV RNA.[16,17] In a study pertaining to meta-analysis of CDV, higher positivity rates were reported from enzyme-linked immuno-sorbent assay, IC and RT-PCR assays,[15] where the lowest observed frequency(25.00%) of detection of CDV was observed in blood samples compared to the overall estimate (37.00%). Low detection rate in RT-PCR in their study was attributed to the differences in the studied populations, period of infection and to the fact that several dogs in their study were presented with neurological impairment and clinical signs present several days in advance, which led to the reduced possibility of finding viral RNA in the plasma even when nested PCR was used. Findings of the present study corroborated the observations of Tavakoli Zaniani *et al.*, in which they detected 29 and one positive cases of CD amongst symptomatic and asymptomatic dogs with immuno-chromatography kits, respectively, while 37 and three CDV-positive cases were detected using RT-PCR among 50 symptomatic and 50 asymptomatic dogs, respectively.[18] The determination of which sample to analyze depends on the method of detection and the opportunity to collect the biological material representative of the evident clinical signs.[5]

In principle, immuno-chromatographic technique (ICT) and RT-PCR methods are different for antigen detection as ICT relies on the capture antibody attached to the gold conjugate, while RT-PCR test is a method of amplifying the target gene with specific primer sets.[9] In the present investigation it is notable that all the suspected samples which were detected positive with immuno-chromatography were found positive in RT-PCR indicating that the IC test was100% specific, highly valuable and reliable screening test for CD though RT-PCR was more sensitive with blood samples. Similarly, Tavakoli Zaniani *et al.*, also found all the 30 positive samples detected by rapid distemper immuno-chromatography test to be positive by RT-PCR test.[18] Comparing the cost benefit factor of the two tests, keeping aside the set-up cost of laboratory PCR assay is cheaper compared to ICT. The ICT kit amounts for nearly double the cost for one test using RT-PCR. The ICT can be used pen-side with a benefit of immediate results, whereas, RT-PCR is labor intensive and time consuming.

It is well understood from the present study that IC kit lacked sensitivity and specificity when nasal and ocular discharges were used for the antigen detection, however, hundred percent specificity was observed when serum samples were used for the detection using IC test. Hence, it is recommended to use serum samples rather than nasal and ocular samples for diagnosis of CDV when using IC test kits which can be further confirmed with RT-PCR.

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Conflict of interest

The authors declare no conflict of interest.

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